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A NUCLEAR MAGNETIC RESONANCE STUDY OF MANGANESE-(II) ION BINDING TO ADENOSINE TRIPHOSPHATE

BY

DENISE SKELLINGTON

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled

"A NUCLEAR MAGNETIC RESONANCE STUDY OF MANGANESE-(II) ION BINDING TO ADENOSINE TRIPHOSPHATE"

submitted by DENISE SKELLINGTON in partial fulfilment of the requirements for the degree of Master of Science.

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TO MY PARENTS

ABSTRACT

An investigation into the nature and sites of interaction between the Mn(II) ion and adenosine 5'-triphosphate (ATP) has been made. Models of possible Mn-ATP complexes have been examined. Techniques used were ^{1}H and ^{31}P nuclear magnetic resonance (NMR) measurements in both the continuous wave mode and the Fourier transform mode.

The longitudinal (T_{1p}) and transverse (T_{2p}) relaxation times of protons H-2, H-8 and H-1' due to the manganese-(II) ions were measured as functions of temperature. The results of these studies show that $T_{1p} = T_{2p}$ for H-8 at all temperatures, whereas $T_{1p} = T_{2p}$ for H-2 below room temperature. All values of T_{1p} or T_{2p} increase at low temperatures, in contrast with the results obtained by Sternlicht et al. (1).

Lam et al. (2) used ^{13}C NMR as a tool to investigate the Mn-ATP system, and also applied their results to data from proton studies carried out by Sternlicht et al. (1). Lam et al. showed that the distance between the metal ion and proton H-2 was much shorter than that predicted by model studies.

Using equations originally proposed by Solomon and Bloembergen (3,4), the percentage of metal ion binding at N-1 is calculated as approximately (2.6 ± 1) %. In order to rationalize this, an $Mn(ATP)_2$ species could be postulated, with one ATP molecule bound to the Mn(II) ion via the triphosphate chain, and the other bound to N-1. The presence of such a complex has not been proven. A more feasible explanation would be to postulate the existence of a 1:1 Mn(II):ATP complex in which a small percentage of ATP molecules in

solution are in the syn-conformation. More research is needed to establish the percentage of syn-conformers present in ATP solutions.

The contributions of the Eigen-Tamm mechanism and the direct ligand exchange mechanism to the ^{31}P NMR linewidths were examined. The results are in acceptable agreement with those predicted on the basis of equations formulated by Kuntz et al. (5).

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CHAPTER ONE

INTRODUCTION

Nucleotides present in cells participate in many fundamental reactions, especially those of intermediary metabolism and energy transfer. They also serve in transferring organic molecules and in oxidation-reduction reactions. Therefore nucleotides have roles in the cell which are varied and of vital importance. Adenosine 5'-triphosphate, ATP is such a nucleotide. Like all nucleotides, it consists of 3 sections (Figure 1):

(1) a nitrogenous base: adenine (6-amino purine);

(2) a 5-carbon sugar: ribose;

(3) phosphate groups: three covalently bonded phosphates.

In 1929, Fiske and Subbarrow (6) first isolated ATP from acid extracts of muscle. A few years later its structure was deduced by degradation experiments and in 1949 Baddiley et al. (7) confirmed it by total chemical synthesis.

The N-1 position on the adenine ring (Figure 1) has a pKa of around 4.0. The three phosphate groups have four dissociable protons. Three proton pKa's are less than 2.0, and the terminal phosphate group has a pKa of approximately 6.7, depending on the prevalent experimental conditions (8,9). Thus at pH 7.0 ATP is a highly charged anion, as the structure in Figure 1 shows. The protons with pKa's of less than 2.0 are completely dissociated at this pH, and the terminal phosphate group is about 75% dissociated.

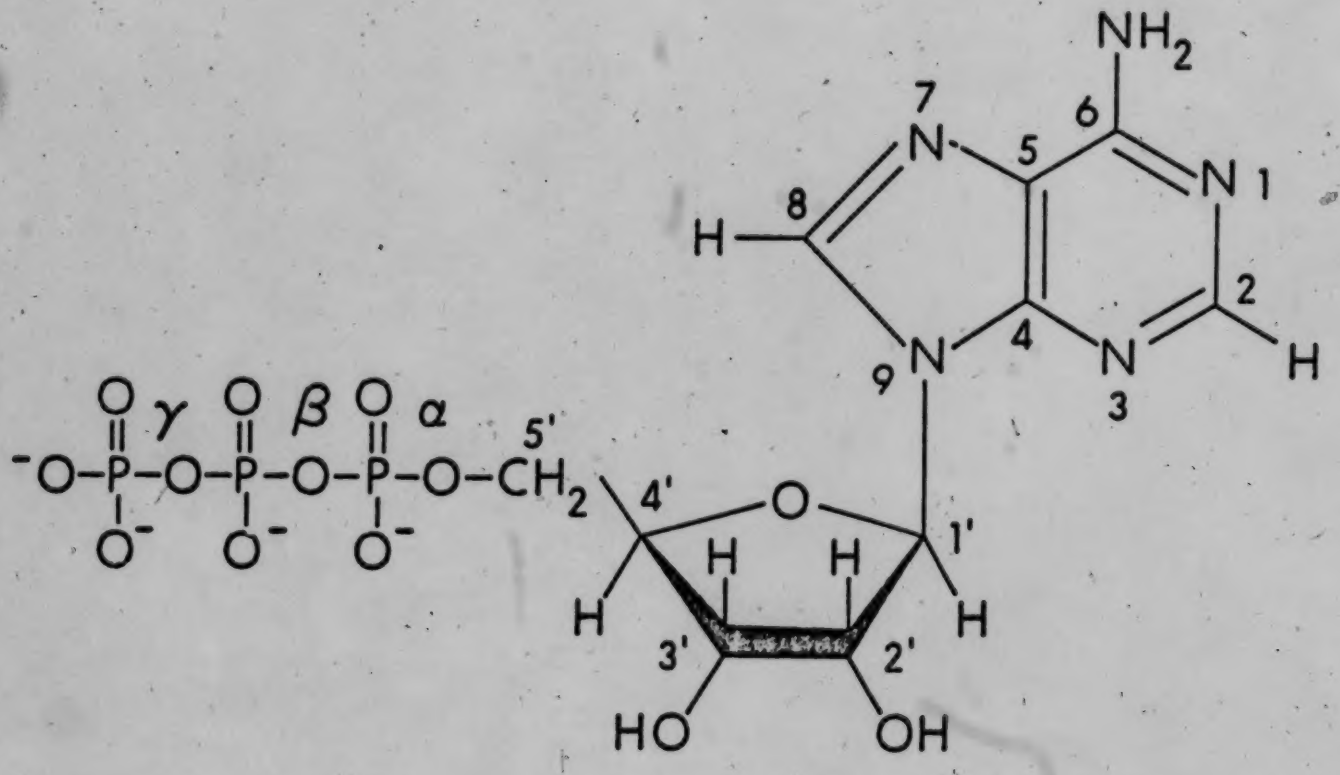

FIGURE 1: Adenosine 5'-triphosphate (ATP).

The adenine base of ATP absorbs at about 260 nm with a high extinction coefficient of 1.57×10^4, which is useful in both the detection and quantitative analysis of ATP. ATP can also be quantitatively analyzed by paper electrophoresis, thin-layer chromatography or ion-exchange chromatography.

Specific enzymes, e.g. creatine kinase (10), are capable of selectively hydrolyzing the third phosphate group without cleaving other bonds. ATP can be hydrolyzed by boiling in 1M hydrochloric acid for at least seven minutes; this removes the second and third phosphate groups, leaving the α-phosphate group intact, but the N-glycosidic link is also broken by this procedure (11).

Even at the time it was first discovered, ATP was suspected of participating in processes of energy transfer within cells, and in 1941 Lipmann (12) postulated that ATP was required whenever processes involving transfer of chemical energy occurred within cells. In fact, the ATP/ADP (adenosine 5'-diphosphate) system is the principal means of cellular energy transfer. This is because ADP is capable of accepting a phosphate group in coupled energy-yielding reactions of metabolism, and the ATP thus formed is capable of donating its terminal phosphate group in coupled energy-requiring reactions.

The last two phosphate bonds of ATP are described as "high-energy phosphate bonds". The term "phosphate bond energy" represents the difference in free energy between products and reactants when a phosphorylated compound is hydrolyzed to inorganic phosphate. This energy, in the case of ATP, is the standard free energy of hydrolysis ΔG°_{hyd} as obtained from the reaction:

$$ATP + H_2O \rightleftharpoons ADP + \text{inorganic phosphate}.$$

Many studies with different enzymes have been made. A value of ΔG°_{hyd} of ATP of -9.5 kcals.mole^{-1} has been established, though some published values differ from this, mainly because measurements were made under different conditions of pH, magnesium ion concentration, etc. (13). It must also be remembered that the standard free energy refers to reactant and product concentrations of 1.0M, which is far from actual cellular conditions. When adjustments are made for true intracellular pH, magnesium ion concentration and steady-state concentrations of ATP, ADP and inorganic phosphate, ΔG°_{hyd} of ATP turns out to be closer to -12.5 kcals.mole^{-1}. Even within a cell, however, this can vary depending on exact intra-cellular conditions at a certain location, i.e. more localized values of pH, etc.

The high concentration of negative charges around the triphosphate group at neutral pH leads to its having a high affinity for cations; the phosphate groups have been shown to complex with divalent metal ions, e.g. Mg^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, etc. (14,15). Because the intracellular concentration of magnesium ions is very high, ATP exists predominantly as an Mg-ATP complex. In fact, in many enzymatic reactions which require ATP, Mg^{2+} ions catalyze the reaction by forming a complex with the substrate (16,17). An adequate explanation of the specificity or advantages of this type of complex has not been found.

The conformation of the Mg-ATP complex has not been exclusively established. In 1957, Szent-Györgi (18) proposed that the metal ion forms a bridge between the triphosphate end of the molecule and the adenine ring. Much research has been done based on this hypothesis, and on the subject of metal ion-adenine ring interactions (16,17). The results have been ambiguous and contradictory, even for the same

metal ions. Methods which have been used include ORD, IR and optical absorption techniques. However, the results of work done with proton NMR shifts and broadening (1,14) have firmly established that at least Zn^{2+}, Cu^{2+}, Ni^{2+}, Co^{2+} and Mn^{2+} ions all interact to some extent with the ring.

Different metal ions form metal-ATP complexes of different structures. The structure of the complex formed with Ni(II) or Co(II) has been established (19,20): there is a bridging water molecule between the metal ion and the adenine ring, and this water probably forms a hydrogen-bond with N-7 while simultaneously the metal ion binds to the three phosphate groups.

In order to determine the nature of the Mg-ATP complex in solution, including distances between the metal ion and various nuclei of the ATP molecule, the NMR approach has been applied. In this case, however, the paramagnetic Mn(II) ion complex with ATP has been studied, since paramagnetic ions have large and specific effects on NMR spectra. These effects are discussed in the following chapter. A study of the Mn-ATP complex may shed much light on the nature of the Mg-ATP complex because it has been shown that Mn(II) ions substitute *in vitro* for Mg(II) ions in many ATP-enzyme catalyzed reactions (21,22).

In a recent paper, Lam *et al.* (2) have applied ^{13}C NMR techniques to the study of the Mn-ATP complex in solution. It was found that for 100% binding of the Mn(II) ions to the oxygen atoms of the three phosphate groups, about 20% of the metal ions were simultaneously bound to the N-7 position of the ring. For the Mn-ATP complex, distances were calculated between the metal ion and the C-4, C-5, C-6 and C-8 carbon nuclei of the base as well as to the H-2 and H-8 protons.

In view of the results from this paper (2), and those of Sternlicht and co-workers (1), it was felt that perhaps the metal ion to proton H-2 distance was rather short. It was therefore thought that there may be some metal-ion interaction with the N-1 nitrogen that could explain this anomaly. The object of the present research is, therefore, to evaluate, on the basis of proton NMR measurements, the degree of interaction of the manganese ion with the N-1 position of the adenine ring.

CHAPTER TWO

THEORETICAL INTRODUCTION

Stability constants of Mn-ATP and Mg-ATP systems were worked out by Mohan and Rechnitz (23). From ion-electrode studies they proved the existence of a Mg_2 ATP species as well as Mg ATP. They carried out measurements on the Mn-ATP system at pH values less than 7.3 since $Mn(OH)_2$ precipitates out at high pH values. Under these and other prevalent experimental conditions, e.g. $Mn^{2+}/ATP \leq 1$, an $Mn(ATP)_2$ structure was not found present in significant concentrations, since the trend in K_{Mn} values was opposite to that expected if $Mn(ATP)_2$ was formed.

The effect of paramagnetic ions on the nuclear longitudinal (T_{1M}) and transverse (T_{2M}) relaxation times has been worked out by Solomon and Bloembergen (3,4). This theory has been extended by Rubinstein et al. (24) but the functional form of the equations remains the same. Thus the calculated relaxation times for the nuclei in the coordination shell of the metal ion are

$$T_{1M}^{-1} = \frac{2}{15}\frac{S(S+1)g^2\beta^2\gamma_I^2}{r^6}\left[3\tau_{c1} + \frac{7\tau_{c2}}{1+\omega_s^2\tau_{c2}^2}\right] + \frac{2}{3}S(S+1)\frac{A^2}{\hbar^2}\left[\frac{\tau_{e2}}{1+\omega_s^2\tau_{e2}^2}\right] \quad 1$$

and

$$T_{2M}^{-1} = \frac{1}{15}\frac{S(S+1)g^2\beta^2\gamma_I^2}{r^6}\left[7\tau_{c1} + \frac{13\tau_{c2}}{1+\omega_s^2\tau_{c2}^2}\right] + \frac{1}{3}S(S+1)\frac{A^2}{\hbar^2}\left[\tau_{e1} + \frac{\tau_{e2}}{1+\omega_s^2\tau_{e2}^2}\right]$$

2

where the symbols g, β and s have their usual meanings, and S = 5/2 for the Mn(II) ion; also

γ_I = magnetogyric ratio of nucleus I;

r = nuclear-electron distance;

ω_s = angular electron precession frequency (rad. $sec.^{-1}$);

A/h = scalar coupling constant (Hz) between the nucleus and the paramagnetic centre. Symbols τ_{c1}, τ_{c2}, τ_{e1} and τ_{e2} are defined thus:

$$\frac{1}{\tau_{c1}} = \frac{1}{\tau_R} + \frac{1}{\tau_{e1}} \qquad 3$$

$$\frac{1}{\tau_{c2}} = \frac{1}{\tau_R} + \frac{1}{\tau_{e2}} \qquad 4$$

$$\frac{1}{\tau_{e1}} = \frac{1}{\tau_M} + \frac{1}{T_{1e}} \qquad 5$$

$$\frac{1}{\tau_{e2}} = \frac{1}{\tau_M} + \frac{1}{T_{2e}} \qquad 6$$

where τ_R = rotational correlation time;

τ_M = lifetime of a ligand in the coordination sphere;

T_{1e} = longitudinal electron spin relaxation time;

T_{2e} = transverse electron spin relaxation time.

Bloembergen and Morgan (25) related an effective electron spin relaxation time, τ_s, to the modulation of the zero-field splitting due to collisions between solvent molecules and the complex, thus:

$$\frac{1}{\tau_s} = B\left[\frac{\tau_v}{1+\omega_s^2\tau_v^2} + \frac{4\tau_v}{1+4\omega_s^2\tau_v^2}\right] \qquad 7$$

where τ_v = collisional correlation time;

B = a constant, dependent on the zero-field splitting parameter.

This equation assumes that τ_v is much smaller than τ_s and that the electron longitudinal relaxation time is much greater than the electron transverse relaxation time. More recently Rubinstein et al. (24) have shown that $\tau_s = T_{1e}$ under these conditions and that otherwise exact expressions for T_{1e} and T_{2e} cannot be given. If τ_s is used as the general electron spin relaxation time, it will be temperature dependent through

$$\tau_v = \tau_0 \exp\left(\frac{E_v}{RT}\right) \qquad 8$$

where E_v = activation energy for the motion characterized by τ_v.

As τ_v decreases, the change in τ_s^{-1} will be positive if $\omega_s^2\tau_v^2 \gg 1$, and negative if $\omega_s^2\tau_v^2 \ll 1$, therefore it can be stated that

$$\tau_s = \tau_s^{\circ} \exp\left(\pm \frac{E_s}{RT}\right) \qquad 9$$

where the plus or minus signs allow for a positive or negative temperature dependence.

The temperature dependence of the ligand lifetime is given by the Eyring equation (26):

$$\tau_M = \frac{h}{kT} \exp\left[\frac{\Delta H^{\ddagger}}{RT} - \frac{\Delta S^{\ddagger}}{R}\right] \qquad 10$$

where $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are the enthalpy and entropy of activation

respectively, and the other terms have their usual meaning.

$$\text{Also, } \tau_R = \tau_R^{\circ} \exp\left[\frac{E_R}{RT}\right] \qquad 11$$

where E_R is the activation energy associated with rotational motion.

Equations 7 to 11 inclusive thus account for the observed temperature dependence of T_{1M}^{-1} and T_{2M}^{-1}.

In the case of Mn(II) ions under the present experimental conditions, $(\omega_s\tau_c)^2 >> 1$ and equations 1 and 2 reduce to (24)

$$T_{1M}^{-1} = \frac{2}{5}S(S+1)g^2\beta^2\gamma_I^2\tau_{c1}r^{-6} \qquad 12$$

$$T_{2M}^{-1} = \frac{7}{15}S(S+1)g^2\beta^2\gamma_I^2\tau_{c1}r^{-6} + \frac{1}{3}S(S+1)\frac{A^2}{\hbar^2}\tau_{e1} \qquad 13$$

From now on, τ_{c1} will be referred to as τ_c. The scalar term indicates two types of interaction, a pseudocontact "through space" interaction, and "leakage" of the unpaired spin density on the Mn(II) ion over to the bound ligand through chemical bonds. These two interactions are detected by the differences in T_{1M}^{-1} and T_{2M}^{-1}.

In the present study, the ATP concentration is very much greater than the concentration of the Mn^{2+} ions, therefore neither the bound linewidth nor the bound longitudinal relaxation time is ever directly observed. What is observed is the concentration-weighted average relaxation time of free and complexed ATP. Swift and Connick (26) relate the observed transverse relaxation rate to the bound transverse relaxation rate and to the NMR exchange rate. Under conditions where $\Delta\omega_M^2 << [\tau_M T_{2M}]^{-2}$, where $\Delta\omega_M$ is the difference in resonance

frequencies between free and bound ATP, their equation for the nuclear paramagnetic transverse relaxation time T_{2p}, is

$$\frac{1}{T_{2p}} = \frac{1}{T_{2,obs}} - \frac{1}{T_{2,0}} = \frac{f}{T_{2M} + \tau_M} \qquad 14$$

where $\frac{1}{T_{2,obs}}$ = transverse relaxation time of metal ion-ligand solution;

$\frac{1}{T_{2p}}$ = transverse relaxation time of pure ligand solution

f = [metal ion]/[ligand]

In the slow chemical exchange region this equation reduces to

$$\frac{1}{T_{2p}} = \frac{f}{\tau_M} \qquad 15$$

and for rapid chemical exchange at high temperatures it becomes

$$\frac{1}{T_{2p}} = \frac{f}{T_{2M}} .$$

These two conditions occur because the temperature dependence of T_{2M}^{-1} generally is less than that of τ_M^{-1}.

Luz and Meiboom's (27) equation for the 2-site exchange involving the longitudinal relaxation time due to the paramagnetic ion is given by

$$\frac{1}{T_{1p}} = \frac{1}{T_{1,obs}} - \frac{1}{T_{1,0}} = \frac{f}{T_{1M} + \tau_M} \qquad 16$$

where T_{1p} = longitudinal relaxation time due to paramagnetic ions;

$T_{1,obs}$ = longitudinal relaxation time of metal ion-ligand solution;

$T_{1,0}$ = longitudinal relaxation time of pure ligand solution.

These equations and principles have been applied by many workers in an attempt to work out the nature of the metal ion-ATP complex in solution.

Cohn and Hughes (14) investigated the effects of metal ions on the proton NMR spectra of nucleotides, looking for interactions between the ion and the adenine ring. They also studied the effects of paramagnetic ions on the ^{31}P NMR linewidths. They interpreted the ^{31}P data as showing that Mg^{2+}, Ca^{2+}, Zn^{2+} and Cu^{2+} ions bind only to the β and γ phosphate groups of ATP. They observed that Mn^{2+} ions broadened all three phosphorus resonances, indicating binding to all three phosphate groups. Their shift studies showed that only proton H-8 in the Zn-ATP complex experienced a noticeable shift. Paramagnetic ions tended to broaden the resonance peaks of protons H-2, H-8 and H-1', especially that of H-8, from which Cohn and Hughes concluded that all paramagnetic ions form a similar complex with the adenine ring. They postulated N-7 as the binding site for these ions, but not for the Mg-ATP complex, as the H-8 resonance in this case did not suffer a shift. They acknowledged that their data was consistent with Szent-Györgi's (18) intramolecular chelate structure, but could not eliminate the possibility of an intermolecular complex.

Sternlicht and co-workers extended these studies on the Mn-ATP system. In their ^{31}P NMR studies (15), the ATP concentration was kept constant at approximately 0.35M. Measurements were made at different metal ion concentrations, and temperature studies were carried out. For the Mn-ATP complex at all temperatures, $fT_{2p} = \tau_M$, i.e.

T_{2p} is in the chemical exchange region. Therefore no structure information could be obtained. T_{2M}^{-1} is scalar-determined and is defined by equation 13. In contrast, $fT_{1p} \approx T_{1M}$, and T_{1M}^{-1} is dipolar-determined, and defined by equation 12. Dilution of the solutions by a factor of a half did not alter the linewidths, nor did reducing the pH to 3.0. There were no observable shifts between pure ATP and Mn-ATP solutions. Within experimental error of about 10% all three ^{31}P linewidths were the same. They also observed that at room temperature the α peak saturated somewhat sooner than the β or γ peaks, and from this they estimated that $T_{1p}|_{\beta,\gamma} \approx \frac{3}{4}T_{1p}|_{\alpha}$. At higher temperatures all three saturated simultaneously and equally. Therefore there was a small difference in the metal ion-phosphate distances. In a unique Mn-ATP complex, these almost equal distances imply simultaneous binding to all three phosphate groups. They calculated values of the hyperfine coupling constants for the three phosphate groups and compared these values with those obtained by Mays (28,29). The agreement of their values with Mays' led them to conclude that the binding to all three phosphate groups was complete.

Further studies on manganese-ATP interactions were made by Brown et al. (30). They measured T_{1p} as a function of temperature and interpreted their results in terms of the dipolar interaction between electron and nuclear spins. They confirmed that the α-phosphate group is more distant from the Mn(II) ion than the β or γ groups, and that the distances of the metal ion from the β or γ groups were the same, at pH 8.5.

Sternlicht et al., in their proton NMR studies of metal ion:ATP binding, measured T_{1p} and T_{2p} as a function of temperature.

The relaxation times for H-8 and H-2 were found to be approximately equal within the experimental uncertainty of 20%. However, for H-1' T_{1p} was 2 to 3 times greater than T_{2p}. These workers professed that H-1' measurements were difficult to do accurately, and differences between T_{1p} and T_{2p} could have been due to experimental errors. They concluded that $fT_{1p} = fT_{2p}$ within experimental uncertainties of 20% for H-2 and H-8, and 100% for H-1'. Therefore $fT_{1p} = T_{1M} = T_{2M} = fT_{2p}$ since the data are in the rapid chemical exchange limit. In the expression for T_{2M}^{-1} (equation 13) the dipolar term must dominate the scalar term. Below room temperature the H-8 resonance narrowed because it had entered the τ_M region. The τ_M values were identical with those obtained from the ^{31}P data (15).

These results were interpreted as showing that whenever a metal ion binds to the triphosphate moiety the metal ion must also bind to the ring; since the Mn^{2+} ion binding to the phosphates is 100%, these authors said that metal ion-ring binding must also be 100%.

Below room temperature proton H-2 and H-1' resonances continued to broaden. The behaviour of H-2 and H-1' was anomalous because these protons had not entered the τ_M region, whereas H-8 had. Sternlicht *et al.* (1) offered no explanation for this anomaly.

The conformation of the Mn-ATP complex in solution could thus be obtained from either T_{1M} or T_{2M} measurements using equation 12 since for proton NMR $T_{1M} = T_{2M}$. Sternlicht and his colleagues (1) chose to use the value of the correlation time τ_c (3×10^{-10} sec) (15) for the water ligands, since the results thus obtained were in better agreement with those for the corresponding Ni^{2+} and Co^{2+} complexes and were consistent with binding near N-1 and N-7. Values of the distance

r between the Mn^{2+} ion and protons H-8, H-2 and H-1' were calculated assuming 100% binding to the ring at one unique site. N-1 was rejected as a possible binding site for three reasons:

(1) No difference was detected in the spectra between measurements at pH 4 and 7. At pH 4, N-1 is protonated, therefore a change might be expected, which was not observed.

(2) Measurements of T_{1p} and T_{2p} for a particular proton gave the same value of τ_M. If there had been a variety of complexes, then different values of τ_M would have been expected. However, studies performed by Lam et al. (2) using ^{13}C NMR and Kuntz et al. (5) using ^{31}P NMR techniques have since shown that different τ_M values are not expected.

(3) The cobalt complex with inosine triphosphate (ITP) gave the same distance results as Co-ATP. In Co-ITP Sternlicht et al. (1) said there was less reason to expect several binding sites, although the results were quite different, and studies by Kuntz and Kotowycz (31) have shown binding of the metal ion to the C=O group and to N-7.

Sternlicht and co-workers (1) discussed possible models for the Mn-ATP complex with regard to their experimental results: the complex was either an intramolecular (back-bound), one metal ion:one ATP molecule complex, or one of various suggested intermolecular complexes. Their results strongly favoured the former.

Since Mn^{2+} ions substitute for Mg^{2+} ions in vitro in enzymatic reactions involving ATP (10,22), the Mg-ATP complex may well be back-bound also at body temperature. Sternlicht et al. (1) felt that the absence of proton chemical shifts in the Mg-ATP complex (14) does not mean that there is no binding to the ring, but only reflects the

insensitivity of the proton chemical shifts to the bound Mg^{2+} ion.

Three years later, Sternlicht and co-workers postulated the presence of a $Mn(ATP)_2$ complex at high total ATP concentration (32). In this complex, they proposed that the metal ion binds to the triphosphate group of one ATP molecule, and to an adenosine nitrogen of the second in a stacked conformation forming what they termed an ApMAr complex. They said that binding to N-7 predominates in such a complex. The results of the ATP/AMP competition studies in the presence of Mn^{2+} ions support the existence of this 1:2 species. At high ATP concentrations (0.02 - 0.5M) the Mn^{2+} ion to proton H-8 distance was found to be 3.5Å and the Mn^{2+} ion to proton H-2 distance was 4.8Å. Hence ApMAr was the simplest complex consistent with these results, with perhaps an ApMAp structure being a minor species based on electrostatic considerations and results from ^{31}P competition studies. T_{2p} for ^{31}P was exchange-controlled at room temperature, i.e. the rate-determining step for relaxation was the rate at which a ligand binds to Mn^{2+} (once binding occurs, relaxation is very rapid). Therefore $T_{2p}^{-1} = f/\tau_M$ and τ_M corresponds to the lifetime of the bound ligand in solution before exchanging chemically with another ligand bound to the metal ion. As the temperature is increased, the exchange rate increases, until $\tau_M < T_{2M}$ and the rapid exchange limit is then reached. Then $T_{2p}^{-1} = f/T_{2M}$, where T_{2M} is the relaxation time of the ligand in the coordination shell of the metal ion.

Recently, however, Wee et al. (33) have re-investigated the Mn-ATP study approach by means of proton NMR and observed a disagreement between measurements and those of Sternlicht et al. (32). This paper is discussed in detail later.

Studies have been carried out on other metal ion-ATP complexes in addition to the Mn-ATP system. Proton studies were carried out by Glassman et al. (19) and Kuntz et al. (20) on ATP complexes with nickel and cobalt ions as well as with manganese ions. The work by Glassman et al. (19) showed that in the Ni(II)-ATP complex there is a water molecule which is bound to the metal ion and is simultaneously hydrogen-bonded to N-7 of the adenine ring. The latter conclusion was derived from a comparison between shift and broadening studies of metal complexes with tubercidin phosphate. In addition the metal ion coordinates to the three phosphate groups.

T_1 and T_2 studies of the solvent water were also carried out by these workers (19). If the ring does not enter the inner coordination sphere of the metal ion, then the ion should still have three water molecules in its inner sphere. These were measured indirectly by comparing with a standard, the Mn^{2+}-cytidine triphosphate (Mn-CTP) complex which contains a known number (three) of complexed water molecules and no ring interactions. For the Mn^{2+} and Co^{2+} complexes it was found that the solvent water proton relaxation times were the same with CTP as with ATP, thus implying no direct metal ion-ring bond. For solutions of Ni-ATP and Ni-CTP, the ratio of water T_1's was 2:3. Glassman et al. suggested two possible explanations for this, that the Ni-ATP complex was either

(1) An inner sphere complex; or

(2) A complex containing a bridging H_2O molecule, with the H_2O exchanging very slowly.

The authors also looked at shifts in a similar Ni^{2+} complex containing a direct Ni^{2+}-N bond, and these shifts were small compared to

those in the Ni-ATP complex, which is strong evidence for explanation (2). Thus they concluded that the Ni-ATP complex contains two fast-exchanging H_2O molecules and one slow (bridging) one.

Kuntz et al. (20) continued this work, looking more closely at the Co-ATP structure through ^{17}O and ^{1}H NMR studies. They investigated the shifts in both Co-ATP and Co-CTP complexes, and found that Co-ATP was 2/3 as effective as Co-CTP in shifting the solvent water signal. This seemed to disagree with previous work (19). However, the results were interpreted to indicate a model for the Co-ATP complex which involves a water molecule coordinated to the phosphate-bound metal ion and this water molecule is also hydrogen-bonded to the ring. Thus for both Ni-ATP and Co-ATP the complexes each contain two fast-exchanging water molecules and one bridging water molecule which exchanges slowly.

The bound water exchange rate in the Mn-ATP system with the ratio Mn^{2+}:ATP approximately equal to unity was measured by Zetter et al. (34). From measurements of the coupling constant A/h they obtained a value of three water molecules in the Mn-ATP system. They suggested that these water molecules were in the inner sphere of the complex and therefore kinetically equivalent, but could not eliminate entirely a structure containing two kinetically equivalent water molecules and a third which exchanges either very rapidly or very slowly.

Zetter et al. (34) compared kinetic parameters for water exchange in the Mn-ATP complex to kinetic data for the substitution of 8-hydroxyquinoline (Ox^-) into the inner sphere of Mn-ATP. The differences were assigned to differences in binding between Mn-ATP

and Ox-Mn-ATP. They also compared kinetic data for the formation of Ox-Mn-ATP and Ox-Mg-ATP complexes. Zetter and co-workers (34) said that some process other than H_2O-exchange was rate-determining in the formation of the Ox-Mn-ATP complex. Ox^- could substitute into Mg-ATP and the rate-determining step would be the loss of H_2O. For Mn-ATP which is more highly substituted, they proposed that a change must take place in the Mn-ATP binding before Ox^- could substitute into the inner sphere. Hence it was postulated that in Ox-Mn-ATP either the ring or α-phosphate group, or both, are no longer bound to the metal. If this were correct, then the structures of the metal ion-Ox-ATP complexes would be almost identical. The interchangeability of Mn^{2+} and Mg^{2+} in enzymatic transphosphorylation could be more easily rationalized in terms of ternary complexes with similar structures, rather than dissimilar structures of binary complexes. Hopes were expressed by Zetter et al. (34) for further investigations into ternary systems; the answers to Mg^{2+}/Mn^{2+} activations lie in these systems, they believed, and not in binary systems.

The most definitive work on the Mn-ATP system was done recently by Lam et al. (2) using natural abundance ^{13}C NMR. They found that all the ring carbon resonances were broadened to some extent by the addition of manganese ions to ATP solutions, with those of carbons C-5 and C-8 being the most broadened, whereas the ribose carbon resonances were unaffected. Data were analyzed in terms of a 1:1 Mn^{2+}:ATP complex, in agreement with the stability constant measurements of Mohan and Rechnitz (23).

The distances between the Mn(II) ion and the carbon nuclei of the base were obtained from the T_{1p} studies. Distance calculations

were also carried out on the proton T_{1p} data obtained by Sternlicht et al. (1). In contrast to the nickel (19) and cobalt (20) complexes in which there is a bridging H_2O molecule between the metal ion and N-7, the Mn-ATP complex had a direct Mn^{2+}-N-7 bond.

Interpretation of the ^{13}C T_{1p} and T_{2p} data led to the following kinetic scheme:

$$M + L \rightleftharpoons ML_p \rightleftharpoons ML_p + r \quad \text{with no } M + L \rightleftharpoons ML_p + r$$

where M = manganese ion;

L = ligand (ATP)

ML_p = Mn-ATP complex with Mn^{2+} bound only to triphosphate moiety;

ML_{p+r} = Mn-ATP complex with Mn^{2+} bound to both triphosphate moiety and to ring at N-7;

or one can say a $\rightleftharpoons$ b $\rightleftharpoons$ c with no a $\rightleftharpoons$ c. Also, [a] >> [b] or [c]. Thus this corresponds to a 3-site exchange. Equations for a 3-site exchange problem have previously been worked-out by Swift and Connick (26) and Angerman and Jordan (35).

T_{1p} and T_{2p} were different because they are governed by different relaxation mechanisms. Under the prevailing experimental conditions, the Solomon and Bloembergen equations become

$$(f'T_{1p})^{-1} = \frac{2}{5}S(S+1)g^2\beta^2\gamma_I^2 r^{-6}\tau_{c1} \qquad 17$$

and

$$(f'T_{2p})^{-1} = \frac{1}{3}S(S+1)\frac{A^2}{\hbar^2}\tau_{e1} \qquad 18$$

where f' = $[Mn^{2+}]$ bound at N-7/total [ATP].

These equations assume that T_{1p} is controlled by dipolar relaxation, but T_{2p} in the rapid exchange region (high temperatures) is controlled by scalar relaxation. Contrast this with the ^{31}P T_{2p} data (15) which are in the slow chemical-controlled region, and ^{1}H T_{2p} data (1), which are determined by the dipolar relaxation.

In the low temperature or chemical controlled region, for example for the ^{31}P data,

$$T_{2p}^{-1} = k_{ab} = \tau_{ab}^{-1} = \frac{[b]}{[a]} \tau_{ba}^{-1} \qquad 19$$

where τ_{ab} = lifetime of species a going to species b;

τ_{ba} = lifetime of species b going to species a;

k_{ab} = rate constant of reaction a → b.

Hence the NMR is sensitive to the exchange rate of the entire molecule.

From their graphs they calculated that

$$f' = 0.2\, f_m$$

where f_m = total $[Mn^{2+}]$/total [ATP]

i.e. that there is not 100% binding of the metal ions to the ring as Sternlicht et al. proposed (1), but only 20% bind there. Sternlicht et al. (1) claimed that the exchange rate between the Mn(II) ion and the ring was the same as the exchange rate between the Mn(II) ion and phosphate groups, i.e. bonds were made and broken simultaneously, but Lam et al. (2) found this not to be so. What Sternlicht and co-workers

saw was a rate-determining interaction of the metal ion with the oxygens of the phosphate groups, followed by a rapid interaction with a ring position (36). Lifetimes obtained from proton resonances only reflect the slower process.

Further proton studies on the Mn-ATP system were performed by Wee et al. (33). They made the assumption that linewidth was a function of viscosity, and from their results of linewidth dependence on temperature, pD and concentration, drew the conclusions that [Mn ATP] was greater than about 25%, with [$Mn(ATP)_2$] less than about 75%.

Their results at pD 8.4 imply that both H-8 protons in the 1:2 complex interact with the manganese ion to the same extent, meaning that they are equidistant from the metal ion. They then erroneously compared this information with the structure of the 1:1 "Mn-ATP" complex studied by Glassman et al. (19) (erroneously because Glassman et al. only elucidated the structure of the Ni-ATP complex), and deduced the presence of two H_2O molecules, bridging between the Mn(II) ion and N-7 of each adenine ring. However, the ^{13}C data obtained by Lam et al. (2) shows that Mn^{2+} ions coordinate directly to N-7.

Detailed kinetics and mechanism of exchange in the Mn-ATP system were worked out recently by Kuntz et al. (5). From ^{31}P studies they identified two NMR exchange mechanisms in solution:

(1) The Eigen-Tamm mechanism, basically

$$M_{aq} + L_{aq} \rightleftharpoons M_{aq}L \rightleftharpoons ML$$

where the reaction $M_{aq} + L_{aq} \rightleftharpoons M_{aq}L$ is diffusion-controlled;

$M_{aq}L \rightleftharpoons ML$ is the rate-determining step;

ML is an inner sphere complex.

The overall reaction is then

$$M_{aq} + L_{aq} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ML$$

where k_1 = forward rate constant for the formation of ML;

k_{-1} = reverse rate constant for the formation of ML.

(2) Direct ligand exchange:

$$ML^* + L \overset{k_2}{\rightleftharpoons} ML + L^*$$

where L* indicates that this ligand has just left the complex;

k_2 = rate constant for direct ligand exchange mechanism.

Considering both mechanisms to operate, expressions for lifetimes are arrived at, viz.:

$$\tau_L^{-1} = k_{-1} \frac{[ML]}{[L]} + k_2[L] \qquad 20$$

$$\text{and } \tau_M^{-1} = k_{-1} + k_2 [L] \qquad 21$$

where τ_L = lifetime of L in state L_{aq} for mechanism 1;

τ_M = lifetime of L in complex ML.

Equation 21 had previously been worked out by Pearson and Lanier (37). In working out the magnitudes of k_1 and k_2, Kuntz et al. studied ATP concentrations between 0.1M and 0.01M. k_1 was calculated to be

$(6\pm1) \times 10^8$ and k_2 $(1.8\pm0.3) \times 10^6$ M^{-1} sec^{-1} for a typical equilibrium constant value of 10^4 M^{-1}.

Recent work by Kuntz and Kotowycz (31) based on ^{13}C T_{1p} on the manganese-inosine 5'-triphosphate system shows the presence of a 1:1 complex with no bridging H_2O molecules. Their T_{2p} studies using ^{13}C NMR indicate two sites of interaction on the hypoxanthine ring, namely N-7 and the carbonyl group. They derive equations for 4-site exchange and discuss the misinterpretation of the data that Wee et al. (33) made in relating proton T_1 measurements of pure ATP solutions to the viscosity of the solutions.

To summarize, it is firmly established that all three phosphate groups in ATP bind virtually 100% of the Mn(II) ions (14,15), and the adenine ring also coordinates with the Mn(II) ions to some extent (14,15,30,32,34). Of the binding sites on the ring suggested, N-1 was dismissed by Sternlicht et al. (1). Lam et al. (2) confirmed N-7 as a binding site, and calculated 20% for the degree of direct coordination.

Various models have been suggested for the Mn-ATP structure (1,2,32,33,34), but the most feasible one to date seems to be the 1:1 complex with no bridging H_2O molecule (2). This is in contrast to the equivalent Ni(II) (19) and Co(II) (20) complexes, which have a H_2O bridge between the metal ion and N-7 of the adenine ring.

In the light of my experimental results, a possible binding site for Mn^{2+} ions at N-1 will be discussed, and other models for the Mn-ATP system will be examined.

CHAPTER THREE

EXPERIMENTAL

ATP (disodium salt, from equine muscle) and adenosine 5'-monophosphate (AMP, sodium salt, from yeast) (both of highest purity), were obtained from Sigma Chemical Co. D_2O solvent was obtained from Columbia Organic Chemical Co. Tetramethyl ammonium chloride (TMA), $N(CH_3)_4Cl$ was obtained from Eastman-Kodak Co. Sodium hydroxide (certified concentrated solution standard), manganese chloride crystals $MnCL_2 \cdot 4H_2O$ and manganese nitrate $Mn(NO_3)_2$ standard solution were obtained from Fisher Scientific Co.

1. Preparation of Solutions

Stock solutions of manganese chloride, about 10^{-2} and 10^{-3}M in Mn(II) ions were prepared in D_2O and were used in the preparation of all subsequent solutions containing metal ions. Using calibrated H. E. Pedersen micropipets, the metal solutions were added to the ATP solutions as required to obtain final solutions with given Mn(II):ATP ratios, i.e. certain values of f_m (see Table I).

A solution of approximately 4 mg/ml of TMA in D_2O was prepared to be used as the reference standard. A solution of ATP in this was prepared, and sufficient $NaOH/D_2O$ solution was added to make the final concentration 0.2M at a pD of 7.4 ± 0.2 (38) (Solution A1). Some of this solution was diluted in the TMA/D_2O solution to prepare a 0.02M ATP solution (Solution B1). The pD of this solution was checked and was in the prescribed range. Initial purification of ATP was not carried out on these particular ATP solutions, nor was extensive replacement of

TABLE I

Summary Of Data On All Solutions

Designation	[ATP], M	f_m	NMR Experiments Performed
A1	0.2		^1H T_2
A2	0.2	2.00×10^{-4}	^1H T_2
A3	0.2	6.00×10^{-4}	^1H T_2
A4	0.2	1.20×10^{-3}	^1H T_2
B1	0.02		^1H T_2
B2	0.02	2.53×10^{-4}	^1H T_2
B3	0.02	1.52×10^{-4}	^1H T_2
B4	0.02	7.09×10^{-3}	^1H T_2
C1	0.17		^1H T_1 and ^1H T_2
C2	0.17	2.35×10^{-4}	^1H T_1 Protons H-8, H-2 and H-1' (Figure 4) ^1H T_2 Proton H-8 (Figure 4)
D1	0.011		^1H T_1
D2	0.011	4.60×10^{-4}	^1H T_1 Protons H-8 and H-1' (Tables II and III)
D3	0.011	2.76×10^{-3}	^1H T_1 Protons H-8 and H-1' (Tables II and III)
E1	0.17		^1H T_1 and ^1H T_2
E2	0.17	1.41×10^{-3}	^1H T_1 Proton H-2 (Figure 4) ^1H T_2 Proton H-8 (Table IV)
E3	0.17	2.25×10^{-3}	^1H T_2 Proton H-2 (Figure 4)
F1	0.16		^{31}P T_2 - all peaks (Figure 5)
F2	0.16	2.50×10^{-4}	^{31}P T_2 - all peaks (Figure 5)
G1	0.016		^{31}P T_2 - all peaks (Figure 5)
G2	0.016	3.17×10^{-4}	^{31}P T_2 - all peaks (Figure 5)

H_2O by D_2O, as the presence of some H_2O is necessary to provide a sufficiently strong lock signal.

Solutions of the following f_m values were prepared from the above ATP solutions by adding the necessary amounts of the stock Mn(II) solutions:

from the 0.2M ATP solution: 2.00×10^{-4}; 6.00×10^{-4}; and 1.20×10^{-3} (solutions A2, A3 and A4 respectively);

from the 0.02M ATP solution: 2.53×10^{-4}; 1.52×10^{-4}, and 7.04×10^{-3} (solutions B2, B3 and B4 respectively).

Since preliminary measurements of transverse relaxation times (T_2) were made on these solutions, the values of f_m were chosen in order to obtain the optimum linewidth for each proton resonance at each concentration of ATP. Measurements on H-8 were optimal at the lowest f_m values, on H-1' at the intermediate f_m values, and on H-2 at the highest f_m values.

ATP used for all other NMR measurements was purified as follows: solid ATP as supplied was dissolved in the minimum amount of water, and passed twice through a Dowex-50 cation exchange resin column to eliminate traces of metal ion. The effluent was evaporated to dryness. The residue was then redissolved in D_2O and again evaporated to dryness. Enough D_2O was then added to the purified ATP to prepare a 0.35M ATP solution. This replacement of H_2O by D_2O is necessary since T_1 measurements are strongly affected by the large proton HDO peak. The solution was stored frozen.

When needed, enough NaOH/D_2O was added to some of the stock 0.35M solution to make the final ATP concentration approximately 0.2M

at a pD of 7.4 ± 0.2 (Solution C1). Some of this solution was diluted in D_2O to prepare an approximately 0.02M ATP solution (Solution D1). The pD of this solution was checked and found to be within the acceptable range. When checked by u.v. spectrometry, the concentrations of solutions C1 and D1 were found to be 0.17M and 0.011M respectively.

Sufficient quantities of the stock Mn(II) solutions were added to solutions C1 and D1 to prepare samples with $f_m = 2.35 \times 10^{-4}$ (Solution C2) and 4.60×10^{-4} (Solution D2) respectively. In addition, a solution of 0.011M ATP containing metal ions, of $f_m = 2.76 \times 10^{-3}$ (Solution D3), was similarly prepared.

Proton T_1 experiments were performed on solutions C1, C2, D1, D2 and D3.

When the concentration of solution C1 had been determined spectroscopically, further solutions were prepared from the stock 0.35M ATP solution, of identical concentration. A solution of pure ATP was prepared, as before, for the blank (Solution E1). In addition, two solutions containing Mn^{2+} ions, one of $f_m = 1.41 \times 10^{-3}$ (Solution E2) and the other of $f_m = 2.25 \times 10^{-3}$ (Solution E3) were prepared.

Proton T_2 measurements were carried out on solutions E1, E2 and E3.

For the measurement of ^{31}P transverse relaxation times, further solutions were prepared from the stock 0.35M solution as described for the proton studies. Pure ATP solutions of strength 0.16M (Solution F1) and 0.016M (Solution G1), and ATP solutions containing Mn(II) ions with $f_m = 2.50 \times 10^{-4}$ (Solution F2) and $f_m = 3.17 \times 10^{-4}$ (Solution G2) respectively, were prepared.

Finally, graphs of $T_{1,0}$ *vs* T^{-1} were plotted for all three

protons for solutions C1 and E1, see Figure 6.

2. Analyses of Solutions

pD measurements were made initially on an Orion 801 digital pH-meter and are accurate to ±0.01 pH units. Later these measurements were carried out on a Fisher Accumet 420 digital pH-meter, and the measurements are also accurate to ±0.01 pH-units. A meter-reading of 7.0 corresponds to a pD of 7.4 (40).

The true ATP concentrations were determined by u.v. spectrophotometry, using a Carey model 15 recording spectrophotometer with 5 mm. quartz spectral cells, by observing the characteristic adenine absorption at 260nm, $\varepsilon_{max} = 1.57 \times 10^4$. Thus the exact values of the ATP concentrations and hence f_m were calculated.

The concentrations of the stock Mn(II) solutions were checked by calibrating against a standard manganese nitrate solution. This was done using the single absorption peak in the phosphorus spectrum of 5'-AMP.

When all the NMR experiments had been completed, various checks were made on the solutions used. The only check made on solutions A1 and B1 used for the preliminary T_2 experiments was thin-layer chromatography (tlc), to check the degree of hydrolysis. This showed that there had been extensive hydrolysis of these solutions. This was probably due to the fact that the samples had been subjected to temperatures of up to and including 80°C and hence the data are not presented in the thesis.

The final pD values of all the remaining solutions were checked. The extent of hydrolysis of the 0.17M ATP solutions was evaluated by running the phosphorus T_2 spectra at room temperature and observing the

peaks due to inorganic phosphate. The proton continuous wave spectra of these solutions at room temperature were also recorded, to determine the degree of replacement of H-8 by D.

3. NMR Experiments

(a) Proton Transverse Relaxation Time (T_2) Measurements

Spectra were recorded on a Varian HA-100-12 or Varian HA-100-15 100 MHz NMR spectrophotometer operating in the continuous wave mode using the proton HOD resonance as the lock signal and TMA as the reference signal. The spectral range studied was 500 Hz downfield from the HOD lock signal. Spectra were taken at various temperatures. The peak under study (H-8, H-2 or H-1') was expanded over a range of either 50 or 100 Hz depending on the width of the signal. The downfield shift of each resonance was calibrated. The linewidths at half-height were measured. The broadening due to the presence of the metal ions, $\Delta\nu_{1/2,m}$ was calculated and $1/f_m T_{2p}$ found using the equation

$$\frac{1}{f_m T_{2p}} = \frac{\pi}{f_m} \{\Delta\nu_{1/2,obs} - \Delta\nu_{1/2,0}\} \qquad 21$$

where T_{2p} = transverse relaxation time;

$\Delta\nu_{1/2,obs}$ = linewidth at half-height of solutions containing Mn^{2+} ions;

$\Delta\nu_{1/2,0}$ = linewidth at half-height of pure ATP solution;

$\Delta\nu_{1/2,obs} - \Delta\nu_{1/2,0} = \Delta\nu_{1/2,m}$.

(b) Proton Longitudinal Relaxation Time (T_1) Measurements

Spectra were recorded on a Varian HA-100-15 100 MHz NMR spectrometer interfaced with a Digital FTS/NMR-3 data system, the Nova 1200 computer and the pulse unit FTS/NMR 400-2, operating in the Fourier

transform mode. The deuterium resonance of the solvent D_2O was used as the lock signal. A reference signal is not necessary when T_1 experiments are performed. For each spectrum, ten free induction decay signals were stored in 2K (2048) data points over a corresponding frequency domain of 500 Hz. [Occasionally 4K (4096) data points were used.] The Digilab system yields 2K data points in the transformed real spectrum by zero filling and carrying out a 4K transform. The two-pulse sequence $(-180° - \tau - 90° - T -)_n$ was used (31,40), where τ is the delay time, T = 25 seconds and n = 10 scans. Values of τ used varied, depending on the experiment.

Spectra were recorded at various temperatures. A set of spectra was obtained at a given temperature for a particular solution by varying the delay time τ from 0 seconds to a maximum value determined by the longest longitudinal relaxation time involved. A typical set of spectra are shown in Figure 2.

For each set of spectra, the normalized height, H_N, of a peak due to a particular proton was measured at each value of τ, and subtracted from the value of H_N at maximum τ, $H_{N(MAX)}$, to obtain the parameter $M = H_{N(MAX)} - H_N$. Graphs of log M vs τ were plotted as in Figure 3, and values of the longitudinal relaxation times calculated from the plots. This analysis is based on the following equation:

$$\ln(H_{N(MAX)} - H_N) = \ln 2H_{N(MAX)} - \frac{\tau}{T} \quad (41) \qquad 22$$

Values of T_{1p} were calculated using equation 16:

$$\frac{1}{T_{1p}} = \frac{1}{T_{1M}} - \frac{1}{T_{1,0}}$$

where T_{1p} = longitudinal relaxation time due to presence of

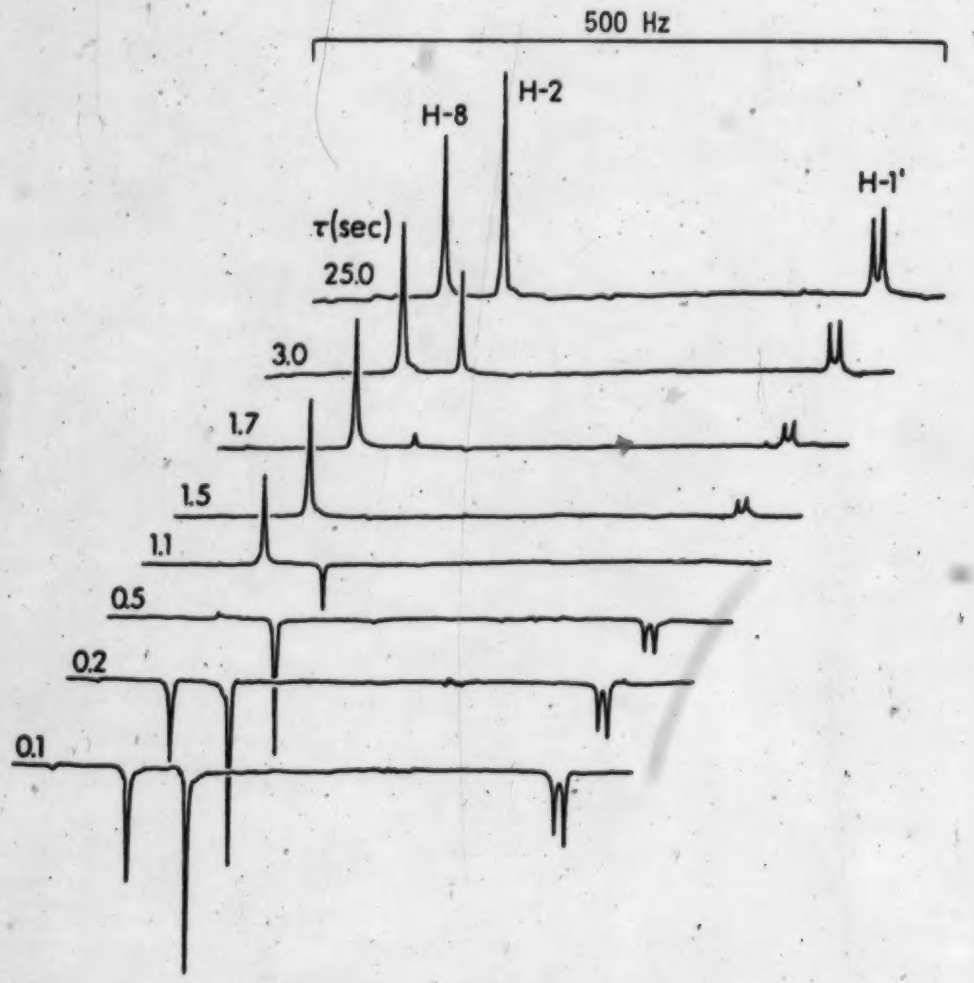

FIGURE 2: A typical series of ^1H T_1 spectra as a function of the τ-values for 0.17M ATP (Solution C1); pD = 7.4 ± 0.2; 318°K.

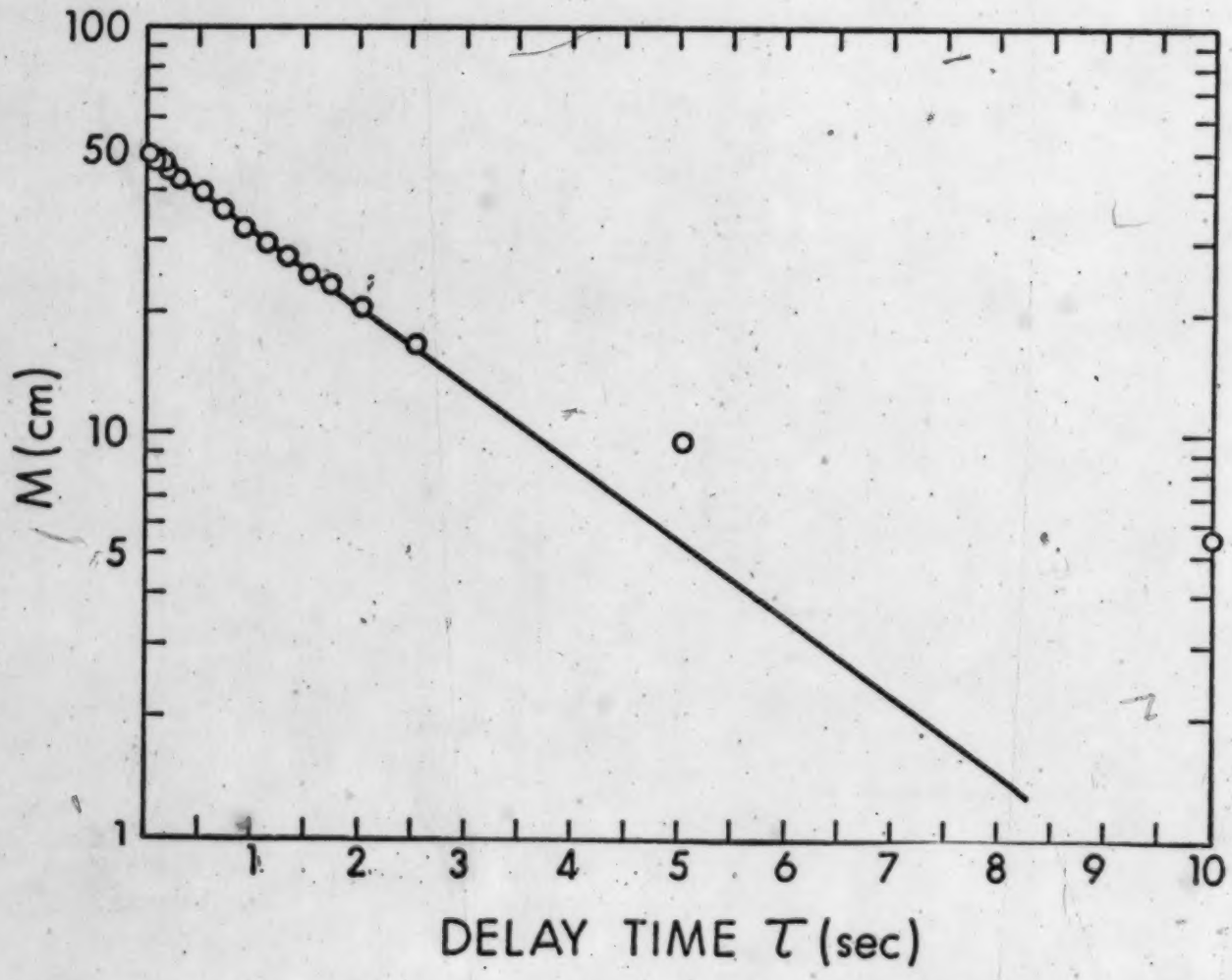

FIGURE 3: A typical plot of M vs τ used to obtain T_1 data for proton H-2 of 0.17M ATP (Solution C1); pD = 7.4 ± 0.2; 318°K.

paramagnetic metal ions;

T_{1M} = longitudinal relaxation time of a solution containing metal ions;

$T_{1,0}$ = longitudinal relaxation time of a pure ATP solution.

(c) Phosphorus Transverse Relaxation Time (T_2) Measurements

Proton noise-decoupled ^{31}P NMR spectra were recorded on an HFX-90 NMR spectrometer (36.4 MHz) interfaced with a Nicolet 1085 data system, operating in the Fourier transform mode. D_2O was used as the lock signal. No reference signal was necessary as only linewidths were measured.

For each spectrum, between 128 and 4K free induction decay signals were stored in 16K or 8K data points, depending on the resolution required, over a corresponding frequency range of 3012 Hz. The Nicolet system then yields 8K or 4K data points in the transformed real spectrum.

Spectra were taken at temperatures within the range 0 to 60°C as seen from Figure 6. Each group of peaks corresponding to the α, β and γ phosphate groups was expanded over a range of 200 Hz. The linewidth at half-height of each peak was measured. At certain temperatures, there was partial collapse of the doublet or triplet structure due to the presence of the metal ions. For these cases a "curve-fitting" procedure was carried out using average values of the coupling constant J obtained from each series of spectra and the true linewidths at half-height were thus obtained. The broadening due to the addition of paramagnetic ions, $\Delta\nu_{1/2,m}$, was calculated, as described for proton T_2 measurements.

(d) Temperature Control

Temperatures were controlled using the Varian and the Bruker temperature control units and were always calibrated before and after an experiment using a thermocouple.

CHAPTER FOUR

RESULTS

Values of the concentrations of the ATP solutions used, values determined by u.v. analysis, have already been given in the previous chapter. f_m values have also been quoted. The pD of all solutions was 7.4 ± 0.2 (38).

The phosphorus T_2 spectra of solutions used for proton T_1 experiments were recorded after all the proton experiments had been completed, and showed there had been virtually no hydrolysis of these solutions.

The integrated proton continuous wave spectra of solutions C1 and C2 were measured at the conclusion of all T_1 measurements. These showed that there had been about 25% replacement of H-8 by D. The same spectra of solutions E1, E2 and E3 showed that there had been over 30% replacement of H-8 by D.

All curves were drawn visually.

Discussion of Errors

The error limits for the proton T_{1p} and T_{2p} measurements shown in Figure 4 are ±10% as optimum values of f_m were used to increase the accuracy of the measurements for each of the protons. The error limits are larger for the T_{1p} data shown in Tables II and III since these measurements were carried out on the 0.011M ATP solutions where the signal-to-noise ratio becomes a problem. In addition, these latter experiments (Tables II and III) were carried out only once.

The T_{2p} data for proton H-2 shown in Table IV is in slight disagreement with the data in Figure 4. The data in Table IV were obtained by carrying out linewidth measurements in the Fourier transform mode and there were phasing and baseline difficulties. However, the general temperature dependence is the same as the T_{2p} data for H-2 shown in Figure 4.

In Figure 6, the scatter of the $T_{1,0}^{-1}$ data for two different samples is likely due to a trace amount of paramagnetic metal ion impurity in the samples which was not removed by the column. However, the straight lines indicate the degree of contamination is very small.

In Figure 5, the ^{31}P T_{2p} data is accurate to ±10%.

TABLE II

Longitudinal Relaxation Time Measurements For H-8 in Solutions D1, D2 and D3

[ATP] = 0.011M

T°K	$10^3/T$ ($°K^{-1}$)	f_m	$T_{1,0}$ (sec)	T_{1M} (sec)	T_{1p} (sec)	$10^{-4}/f_m T_{1p}$ (sec^{-1})
278	3.60	4.60×10^{-4}	0.21	0.185	1.6	0.14
307	3.26	4.60×10^{-4}	0.66	0.118	0.14	1.6
323	3.10	4.60×10^{-4}	0.98	0.140	0.16	1.4
278	3.60	2.76×10^{-3}	0.21	0.080	0.13	0.28

TABLE III

Longitudinal Relaxation Time Measurements For H-1' In Solutions D1 and D2

[ATP] = 0.011M; $f_m = 4.60 \times 10^{-4}$

T°K	$10^3/T$ (°K^{-1})	$T_{1,0}$ (sec)	T_{1M} (sec)	T_{1p} (sec)	$10^{-4}/f_m T_{1p}$ (sec^{-1})
278	3.60	1.28	0.560	0.998	0.22
307	3.26	2.55	1.03	1.73	0.13
323	3.10	3.35	2.00	4.96	0.044

TABLE IV

Tranverse Relaxation Time Measurements For H-2 In Solutions

E1 and E2; [ATP] = 0.17M

T°K	$10^3/T$ ($°K^{-1}$)	$\Delta\nu_{1/2,obs}$ (Hz)	$\Delta\nu_{1/2,0}$ (Hz)	$10^{-4}/f_m T_{2p}$ (sec^{-1})
275	3.64	18.8	2.3	3.7
281	3.56	16.8	2.1	3.3
288	3.47	12.8	1.6	2.5
299	3.34	8.4	1.3	1.6
308	3.25	6.4	1.1	1.2
319	3.13	4.7	1.1	0.80
323	3.10	4.4	1.2	0.71
328	3.05	4.0	1.2	0.62
332	3.01	3.3	1.1	0.49

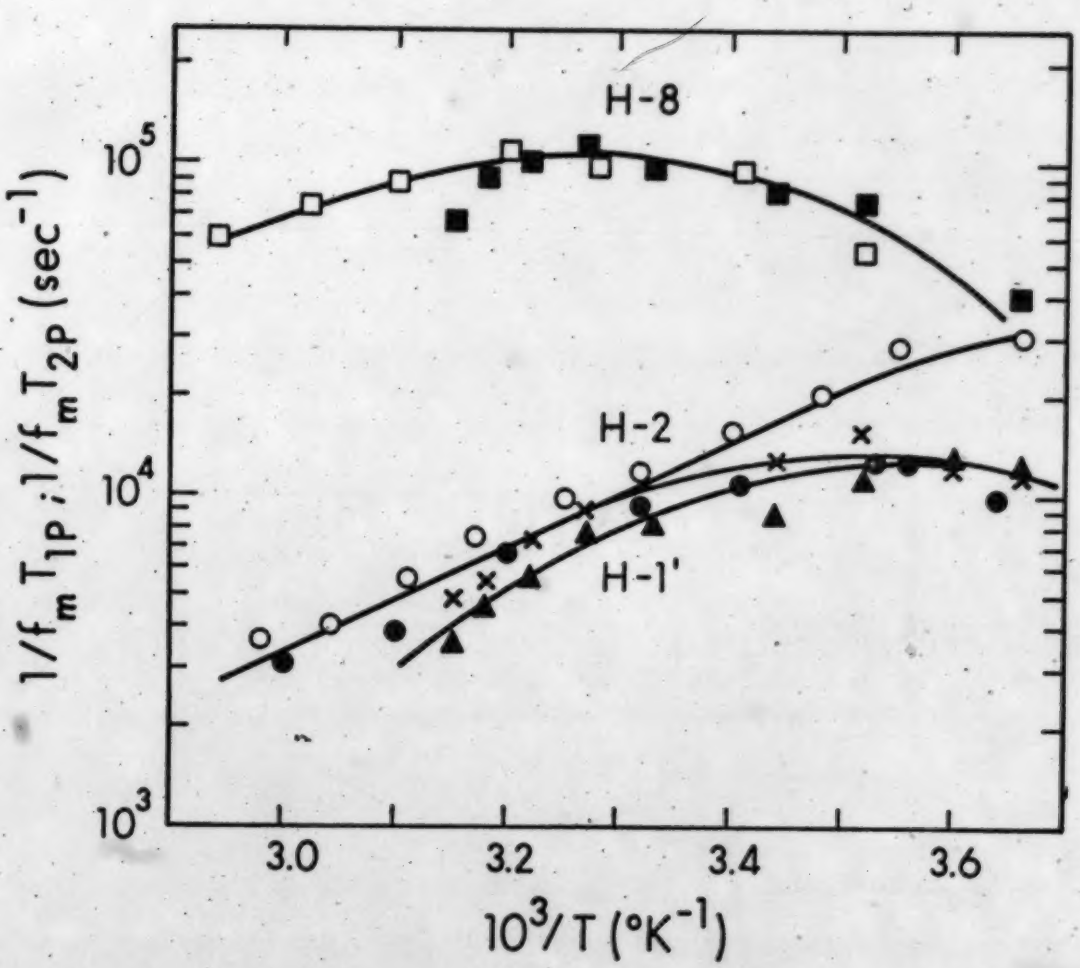

FIGURE 4: A plot of $(f_m T_{1p})^{-1}$ and $(f_m T_{2p})^{-1}$ vs T^{-1} for protons H-8, H-2 and H-1' for 0.17M ATP; pD = 7.4 ± 0.2.

LEGEND
- ■ $(fT_{1p})^{-1}$ for H-8
- □ $(fT_{1p})^{-1}$ for H-8
- X $(fT_{2p})^{-1}$ for H-2
- ▲ $(fT_{1p})^{-1}$ for H-1'

} $f_m = 2.35 \times 10^{-4}$

- ● $(fT_{1p})^{-1}$ for H-2; $f_m = 1.41 \times 10^{-3}$
- O $(fT_{2p})^{-1}$ for H-2; $f_m = 2.25 \times 10^{-3}$

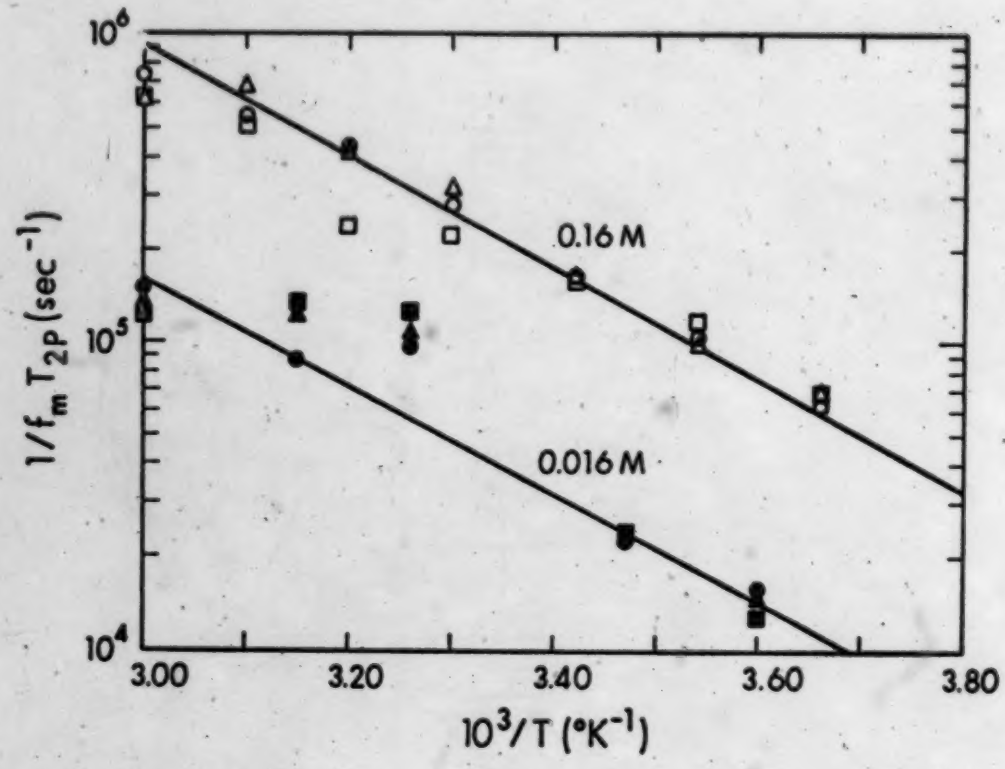

FIGURE 5: A plot of T_{2p}^{-1} vs T^{-1} for the ^{31}P NMR spectra of ATP; pD = 7.4 ± 0.2.

LEGEND

	0.16M ATP Solns. F1 & F2	0.016M ATP Solns. G1 & G2
α - phosphate group	△	▲
β - phosphate group	□	■
γ - phosphate group	○	●

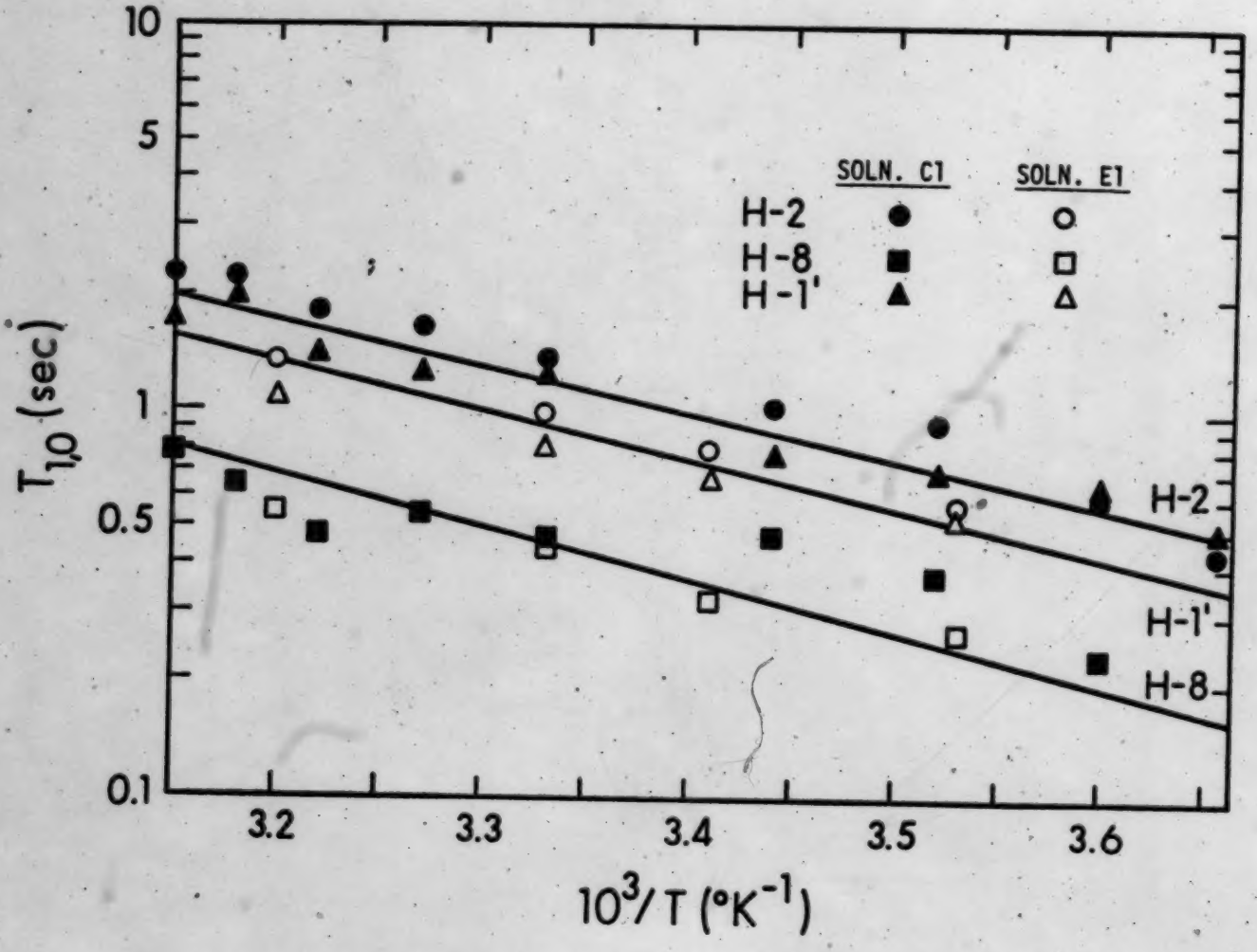

FIGURE 6: A plot of $T_{1,0}$ vs T^{-1} for protons H-8, H-2 and H-1' for 0.17M ATP; pD = 7.4 ± 0.2.

CHAPTER FIVE

DISCUSSION

It is well-established that virtually 100% of the Mn^{2+} ions bind to the phosphate groups of ATP as shown by the large stability constants for this complex (9,10), and the experimental conditions used. From proton NMR studies Sternlicht et al. (1) concluded that the metal also binds to the adenine ring at a position in space between the amino group on carbon C-6, and the nitrogen N-7. They state that the bonds to the ring and to the phosphates are made and broken simultaneously.

Lam et al. (2) have shown, from ^{13}C NMR studies, that the metal ion binds directly at N-7 of the adenine ring, and they calculate the percentage of ions bound there as approximately 20%. They explained the discrepancy with the results of Sternlicht and co-workers (1) by invoking Frey and Stuehr's (36) reasoning, which was the suggestion that what Sternlicht and his colleagues (1) obtained were lifetimes due to an initial, rate determining interaction of the metal ion with the oxygen atoms of the phosphate groups. Then, Frey and Stuehr say, there is a second, rapid step in which the metal ion interacts with a ring position. Therefore Lam et al. (2) postulated a consecutive binding mechanism:

$$a \rightleftharpoons b \rightleftharpoons c$$

where a = pure ATP;

b = ATP with metal ion bound at phosphate groups;

c = ATP with metal ion bound at phosphate groups and ring.

Lam et al. (2) show that the recalculated metal ion to proton H-2 distance (4.4Å) using Sternlicht's (1) data for the complex in which the metal ion binds only at N-7 is much shorter than that of a model study (6.8Å). Other experimental Mn^{2+} ion to carbon nuclei or Mn^{2+} ion to proton H-8 distances were in excellent agreement with those predicted by the model. Therefore it was felt that there was some Mn(II) ion binding at N-1 that only proton H-2 NMR data could detect. (Binding at N-3 was not considered feasible, since in the syn conformation, the N-3 nitrogen is not accessible to the metal ion.) In the present study, an attempt is made to calculate the percentage of metal ions bound to a second ring position, namely N-1.

As was done by Lam et al. (2), equation 12 is employed in the analysis of the proton T_{1p} data shown in Figure 4, namely

$$(f'' T_{1p})^{-1} = \frac{2}{5} S(S+1) g^2 \beta^2 \gamma_I^2 r^{-6} \tau_c ,$$

where f'' = [ATP] bound at N-1/total $[Mn^{2+}]$,

and all other terms have been previously defined. The constants are as follows:

S = 5/2 for the Mn(II) ion;

g = 2.00 (free electron);

β = 9.27×10^{-21} erg gauss^{-1};

γ_I = 2.675×10^{4} rad. sec^{-1} gauss;

r = 3.4×10^{-8} cm. (Mn(II) ion to proton H-2 distance);

τ_c = 7.5×10^{-10} sec.

The value quoted for r is actually that calculated by Lam

et al. (4) for the Mn^{2+} ion to proton H-8 distance. In this study it is assumed that this is the value of the Mn^{2+} ion to proton H-2 distance.

The value of τ_c for the present ATP concentration of 0.17M was arrived at by taking a weighted average between the value obtained by Brown et al. (30) for 0.1M ATP at 20°C (6×10^{-10} sec.) and that by Sternlicht et al. (1) for approximately 0.35M ATP at room temperature (1×10^{-9} sec.).

Solving the above equation using these values one obtains

$$(f''T_{1p})^{-1} = 4.18 \times 10^{5} \text{ sec}^{-1}.$$

From Figure 4, a value of $(f_m T_{1p})^{-1}$ for proton H-2 at 300°K is 1.10×10^{4} sec.$^{-1}$, so that

$$\frac{f''}{f_m} = 0.026 .$$

Thus (2.6 ± 1)% is the extent of binding of Mn(II) ions at N-1. A contribution to the H-2 T_{1p}^{-1} value due to the Mn-ATP complex in which the Mn^{2+} ion binds to N-7 was calculated using equation 12 and a value of r = 6.8Å. This complex contributes less than 10% to the observed T_{1p}^{-1} of H-2 and therefore this correction was not made.

This same analysis is applied to the proton H-8 T_{1p} data in Figure 4 to consider the binding at N-7, using the value of $(f_m T_{1p})^{-1}$ for H-8 at 300°K of 1.44×10^{5} sec.$^{-1}$. Hence

$$\frac{f'}{f_m} = 0.34$$

This analysis indicates that about 34% of the metal ions bind at N-7,

in disagreement with the calculations of Lam et al. (2). However, in this calculation a tangent must be drawn in the high temperature region for the proton H-8 T_{1p} and T_{2p} data. The value of 1.44×10^5 sec.$^{-1}$ is obtained by extrapolation, and is subject to a large error. For proton H-2, however, the T_{1p} and T_{2p} data are well into the fast-exchange region at 300°K.

The next question that must be considered is whether the ^{13}C NMR measurements could detect such a low degree of binding at N-1. Lam et al. (2) did not see an effect of the Mn(II) ions on the ^{13}C T_1 data of carbon C-2.

This can be analyzed in the following manner:

First, estimate the Mn(II) ion to carbon C-2 distance as 3.1Å, the same as the Mn(II) ion to carbon C-8 distance. Equation 12 is then applied, and using the value for γ_I of ^{13}C of 6.73×10^3 rad. sec.$^{-1}$ gauss, one obtains

$$(f''T_{1p})^{-1} = 4.6 \times 10^4 \text{ sec}^{-1}$$

Now, for 2.6% binding at N-1, one can calculate that for $f_m = 2.35 \times 10^4$, a value used in the present study and within the range covered by Lam et al. (2), the value of T_{1p} is 0.28 secs. This value was not detected by Lam et al.

Finally, how can one rationalize a small degree of manganese ion binding at N-1? First of all, nucleotides (42) including ATP (1,43, 44) have been shown to self-associate or base-stack. An $Mn(ATP)_2$ complex was originally postulated by Sternlicht et al. (32), and in their model the metal ion was bound to the phosphate groups of one ATP molecule and to the N-7 of the neighboring ATP molecule. This complex

was discarded by Wee et al. (33). In addition, an $Mn(ATP)_2$ complex was not detected by Mohan and Rechnitz (23).

Wee et al. (33) postulated the existence of a 1:2 complex containing two water molecule bridges between the Mn(II) ion and N-7 positions of both ATP molecules. The metal ion was simultaneously bound to the triphosphate chain of one ATP molecule. However, Lam and co-workers (2) show that Mn(II) ions coordinate directly to N-7.

Secondly, ATP exists largely in the anti-conformation as shown in Figure 1. Therefore if one considers the possibility of a 1:1 metal ion:ATP complex, some ATP molecules must be present in solution in the syn-conformation in order for the metal ion to bind at N-1.

An $Mn(ATP)_2$ complex may be considered a minor species, in such low concentrations that it has not yet been detected. In such a complex there would be no water molecule bridges; one ATP molecule must have its phosphate groups bound to the metal ion, and N-1 of the second ATP molecule is also bound. In this structure both ATP molecules would be in the anti-conformation. Such a species, of course, cannot be present in significant amounts.

If we say that the small amount of Mn(II) ions binding at N-1 is due to both of these factors to differing extents, then any method to detect either of these would have to be extremely sensitive. Certainly, a small fraction of syn-conformers could not be readily detectable, and Sternlicht et al. (1) failed to detect any binding at N-1.

The data in Figure 4 can be compared with the data in the paper by Sternlicht and his colleagues (1). It seems that the present data for T_{1p} of H-1' is in disagreement with theirs. The plot in

Figure 4 bends over at low temperatures whereas that of Sternlicht et al. does not; also, in the present study, the plot for H-1' is very close graphically to that for H-2, whereas the results of Sternlicht et al. show that the plots for H-1' and H-2 are well-separated. But it must be noted, and Sternlicht et al. also pointed out, that there may be large errors of up to 100% in their measurements concerning H-1' due to experimental difficulties.

It is seen from Figure 4 that $T_{1p} = T_{2p}$ for proton H-8, and for proton H-2 at and above room temperature. For proton H-2 below room temperature the curves for T_{1p} and T_{2p} diverge and $T_{1p} > T_{2p}$. The data indicates that T_{2p} is in the fast-exchange region while T_{1p} has entered the slow-exchange region. This anomaly cannot be explained.

It is also interesting to see that the plots for T_{1p} of proton H-2 and H-1' in Figure 4 bend over at low temperatures, which they do not do in the paper by Sternlicht et al. (1).

By performing proton T_{1p} and T_{2p} measurements at a low ATP concentration, around 0.02M, it was hoped that a change in the degree of binding at N-1 could be seen. Unfortunately, the studies at such a low ATP concentration proved difficult to perform in practice and the results were inconclusive.

The straight lines in Figure 6 show that the data is self-consistent and that the samples were reasonably pure; metal ion impurities would have given rise to curvature in the plots.

The ^{31}P data in Figure 5 will be examined in the light of the theory developed by Pearson and Lanier (37), and Kuntz et al. (5), and equation 21 will be applied, namely

$$\tau_M^{-1} = k_{-1} + k_2 [L].$$

TABLE V

Calculated and Experimental Values of τ_M^{-1} for 0.16M (F1 and F2) and 0.016M (G1 and G2) ATP Solutions Obtained from ^{31}P NMR Data

	τ_M^{-1} (calculated) sec.$^{-1}$	τ_M^{-1} (experimental) sec.$^{-1}$
0.16M ATP	3.48×10^5	2.32×10^5
0.016M ATP	8.88×10^4	4.25×10^4

All symbols were defined in the Theoretical Introduction. The results of this analysis are given in Table V. It is seen that for the 0.16M ATP solutions there is a factor of about 0.7 difference between the calculated and experimental τ_M^{-1} values, and for the 0.016M ATP solutions this factor is about 0.5. However, Pearson and Lanier (37) point out that the graph of τ_M^{-1} vs ligand concentration is linear only over a limited ligand concentration range and the curve levels off at higher concentrations. This is typical of the kinetics of the present study. The work performed by Kuntz et al. (5) was carried out using ATP concentrations between 0.01 and 0.1M, and the present work was carried out on 0.016M and 0.16M ATP solutions. Possibly the 0.16M ATP data are beyond the linear region.

There are several possible reasons for the differences between the present data and that calculated using the above equation:

(1) Kuntz et al. (5) performed their experiments at a constant ionic strength of 1.0, whereas in the present study the ionic strength was not controlled;

(2) The pD values are different;

(3) Equation 21 assumes binding only to the phosphate groups, with no ring binding.

In view of these differences, the agreement of the data is considered acceptable.

It was hoped that a study of the T_{1p} temperature dependence of phosphorus NMR at a high and a low ATP concentration would lead to accurate values of the correlation time τ_c since the Mn^{2+} ion to P distance is known from X-ray studies for $LiMnPO_4$ crystals (3.3Å) (28,29).

However, the equipment necessary to perform such studies was not available, and τ_c was obtained by taking the weighted average of the correlation time based on the work of Brown et al. (30) and Sternlicht et al. (1).

In conclusion, I should like to postulate a small but not insignificant amount of metal ion binding to the adenine ring at N-1. This may possibly be due to some $Mn(ATP)_2$ species, but more likely due to an Mn ATP species in which the ATP molecule is in the syn-conformation, and the metal ion is bound to both the triphosphate moiety and N-1 of the adenine ring.

Further work in this field could include measurements of the percentage of syn-conformers present in ATP solutions at different ATP concentrations, in order to elucidate the nature of the metal ion binding at N-1. Also, accurate determinations of τ_c should be carried out by performing T_{1p} studies on ^{31}P NMR for each ATP concentration.

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